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(54) Title: INTERLEUKIN-18 MUTANTS, THEIR PRODUCTION AND USE

(57) Abstract: The invention provides mutants of IL-18 with lower affinity to IL-18BP than the wild type IL-18 molecule.

INTERLEUKIN-18 MUTANTS, THEIR PRODUCTION AND USE

FIELD OF THE INVENTION

The present invention relates to IL-18 mutants having enhanced biological activity with respect to the wild type protein.

BACKGROUND OF THE INVENTION

In 1989, an endotoxin-induced serum activity that induced interferon-γ (IFN-γ) obtained from mouse spleen cells was described (Nakamura et al., 1989). This serum activity functioned not as a direct inducer of IFN-γ but rather as a co-stimulant together with IL-2, IFN-/, TNF or mitogens. An attempt to purify the activity from post-endotoxin mouse serum revealed an apparently homogeneous 50-55 kDa protein (Nakamura et al., 1993). Since other cytokines can act as co-stimulants for IFN-γ production, the failure of neutralizing antibodies to IL-1, IL-4, IL-5, IL-6, or TNF to neutralize the serum activity suggested it was a distinct factor. In 1995, the same scientists demonstrated that the endotoxin-induced co-stimulant for IFN-γ production was present in extracts of livers from mice preconditioned with P. acnes (Okamura at al., 1995). In this model, the hepatic macrophage population (Kupffer cells) expand and in these mice, a low dose of bacterial lipopolysaccharide (LPS), which in non-preconditioned mice is not lethal, becomes lethal. The factor, named IFN-γ -inducing factor (IGIF) and later designated interleukin-18 (IL-18), was purified to homogeneity from 1,200 grams of *P. acnes*-treated mouse livers. Degenerate oligonucleotides derived from amino acid sequences of purified IL-18 were used to clone a murine IL-18 cDNA (Okamura et al. 1995). Messenger RNAs for IL-18 and interleukin-12 (IL-12) are readily detected in activated macrophages. IL-18 does not induce IFN-γ by itself, but functions primarily as a co-stimulant with mitogens or IL-12. The human cDNA sequence for IL-18 was reported in 1996 (Figure 1A SEQ ID NO:1).

Interleukin IL-18 shares structural features with the IL-1 family of proteins (Nakamura et al., 1993; Okamura et al., 1995; Ushio et al., 1996; Bazan et al., 1996). Unlike most other cytokines, which exhibit a four-helix bundle structure, IL-18 and IL-1β have an all β-pleated sheet structure (Tsutsui et al., 1996). Similarly to IL-1β, IL-18 is synthesised as a biologically inactive precursor (proIL-18), lacking a signal peptide (Ushio et al., 1996). The IL-1β and IL-18 precursors are cleaved by caspase 1 (IL-1β-converting enzyme, or ICE), which cleaves the precursors after an aspartic acid residue in the P1 position. The resulting mature cytokines are readily released from the cell (Ghayur et al., 1997 and Gu et al., 1997).

IL-18 is a co-stimulant for cytokine production (IFN-γ, IL-2 and granulocyte-macrophage colony stimulating factor) by T helper type I (Th1) cells (Kohnoet al., 1997) and also a co-stimulant for FAS ligand-mediated cytotoxicity of murine natural killer cell clones (Tsutsui et al., 1996).

Th1 lymphocytes are involved in the immune responses against tumors (Seki et al., 2000). Thl responses include the secretion of the cytokines IL-2, 1L-12, IL-18 and IFN-γ, as well as the generation of specific cytotoxic T lymphocytes recognizing specific tumor antigens. The Thl response is also a vital arm of host defence against many microorganisms. However, the Thl response can also be associated with non-desirable effects such as the development of several autoimmune diseases, inflammation and organ transplant rejection.

Attempts to express the mature form of IL-18 in *E. coli* using a vector encoding the mature protein did not provide a fully active cytokine. An efficient expression system for the generation of the fully biologically active human IL-18 has been developed for therapeutic uses, e.g. in malignancies, or any condition where IFN-γ induction is desired (WO 00/61768). In this system, the IL-18 precursor caspase-1 cleavage site has been changed to a factor Xa site (ICE/Xa), and a vector encoding IL-18 ICE/Xa precursor was used for transformation of *E. coli*. Following expression of this IL-18 precursor in *E. coli* the mature IL-18 was generated by factor Xa cleavage *in vitro*. This mature IL-18 generated by factor Xa cleavage was fully active.

Cytokine binding proteins (soluble cytokine receptors) are usually the extracellular ligand binding domains of their respective cell surface cytokine receptors. They are produced either by alternative splicing or by proteolytic cleavage of the cell surface receptor. These soluble receptors have been described in the past, for example, the soluble receptors of IL-6 and IFN-γ (Novick et al., 1989), TNF (Engelmann et al., 1989; Engelmann et al., 1990), IL-1 and IL-4 (Maliszewski et al., 1990), IFN-α/β (Novick et al., 1994; Novick et al. 1992). One cytokine-binding protein, named osteoprotegerin (OPG, also known as osteoclast inhibitory factor-OCIF), a member of the TNFR/Fas family, appears to be the first example of a soluble receptor that exists only as a secreted protein (Anderson et al., 1997; Simonet et al., 1997; Yasuda et al., 1998).

An IL-18 binding protein (IL-18BP) was affinity purified, on an IL-18 column, from urine (Novick et al., 1999). IL-18BP abolishes IL-18 induction of IFN-γ and of IL-8, activation of NF-kB *in vitro* and induction of IFN- *in vivo*. IL-18BP is a soluble circulating protein which is constitutively expressed in the spleen, and belongs to the immunoglobulin superfamily. The most abundant IL-18BP isoform, the spliced variant isoform a, exhibits a high affinity for IL-18 with a rapid on-rate and a slow off-rate, and a dissociation constant (Kd) of approximately 400 pM (Kim et al., 1999).

The residues involved in the interaction of IL-18 with IL-18BP have been described through the use of computer modelling (Kim et al., 1999) and based on the interaction of IL-1 with the IL1R type I (Vigers et al., 1997). In the model for IL-18 binding to the IL-18BP, the Glu residue at position 42 the and Lys residue at position 89 of IL-18 have been proposed to bind to Lys-130 and Glu-114 in IL-18BP, respectively (Kim et al., 1999).

IL-18 is constitutively present in many cells (Puren et al., 1999) and circulates in healthy humans (Urushihara et al., 2000). The high affinity of IL-1BP to IL-18 as well as the high concentration of IL-18BP found in the circulation (20 fold molar excess over IL-18), represents a unique situation in cytokine biology. Therefore, most, if not all, of the IL-18 molecules in the circulation is bound to the IL-18BP.

The circulating IL-18BP which competes with cell surface receptors for IL-18, may act as a natural anti-inflammatory and an immunosuppressive molecule.

Viral agents encode IL-18BP like proteins, for example, *M. contagiosum* viral proteins MC53 and MC54 share a significant homology to mammalian IL-18BP (Novick et al. 1999). *M. contagiosum* proteins MC53 and MC54 possess the ability to bind and neutralize human IL-18 in a fashion similar to that of IL-18B (Xiang and Moss, 1999). The ectromelia poxvirus p13 protein, which is homologous to IL-18BP, binds human IL-18 and inhibits its activity in vitro. Mice infected with a p13 deletion mutant virus exhibited decreased levels of infectivity (Born et al., 2000). Therefore infectivity degree seems to correlate with the presence of IL-18BP.

The high levels of circulating IL-18BP may represent a natural defence against a runaway Th1 response to infection and development of autoimmune diseases. However, IL-18 contributes to the Thl response which is important in host defence against tumors. Therefore, IL-18BP may bring about failure of the host to develop cytotoxic T cells directed against tumor cells. Indeed, there is evidence that IL-18 promotes host defence against tumors in mice. For example, in syngeneic mice, murine mammary carcinoma cells expressing murine IL-12 or murine IL-I8 were less tumorigenic and formed tumors more slowly than did control non-expressing cells (Coughlin et al., 1998). Antibody neutralisation studies revealed that the antitumor effects required IFN-γ. In another study, systemically administration of IL-18 to experimental animals in combination with B16 melanoma expressing B7-1 (CD80) resulted in dramatic suppression of melanoma formation, tumor growth, and a significant improvement in survival (Cho et al., 2000).

Cytokines are used as adjuvant to increase the effectivity of immunotherapy in cancer. For example, IL-2 is administered for renal cell carcinoma or melanoma (Gollob et al., 2000). Often, one important consequence of the treatment with cytokines is severe systemic toxicity profiles. Using cytokines, expressed by the patient's own tumor or dendritic cells, is a logical solution to the problem. Yet, if IL-18 will to be used locally, as adjuvant in tumor immunotherapy, the ability of the constitutive levels of IL-18BP to neutralize IL-18 in the local environment would still be exerted and consequently its effectivity is greately diminished.

The use of non-myeloablative allogeneic transplants, the so-called mini transplants, to treat leukaemia and solid tumors is increasingly successful in inducing graft-versus-leukaemia and graft-versus-tumor reactions (Slavin S., 2000; Slavin et al., 2000). Two studies that used either allogeneic peripheral blood stem cells (Childs et al. 2000) or dendritic cells (Kugler et al., 2000) to treat patients with metastatic renal cell carcinoma met a remarkable success. Although these studies need to be extended and confirmed, the concept that an ongoing graft-versus-tumor reaction is exploitable for immunotherapy in cancer is gaining acceptance (Slavin, 2000). Since IL-18 appears to be involved in these successful therapeutic approaches, a further improvement may be achieved if the neutralizing effect of IL-18BP can be abolished.

Mutants of IL-18 (IFN-γ, inducing factor) are described in EP0845530. The described IL-18 mutants are molecules in which 1, 2, 3 or all 4 cysteine residues in IL-18 (Figure 1B) were replaced by serine or alanine residues. These mutants contained an intact consensus sequence (Figure 1B). All the isolated mutants exhibit higher stability than wild type IL-18. The degree of stability of the mutants is directly proportional to the number of Cys residues replaced in the molecule. EP0845530 is silent on the ability of IL-18BP to neutralize these mutants.

The generation and therapeutic use of fully active IL-18 mutants unable to bind or bind with low affinity to IL-18BP, is therefore highly advantageous.

SUMMARY OF THE INVENTION

The present invention relates to an IL-18 mutant polypeptide, comprising mutations in one or more amino acid residues which are involved in its interaction with IL-18 binding protein. More specifically, the mutations are substitutions, preferably non-conservative, additions or deletions. The residues mutated in said polypeptide may be selected from Glu-42, Ile-85, Met-87, Lys-89, Met-96, Asp-130, Lys-132, Pro-143, Met-149, and Leu-189, preferably, Glu-42 and Lys-89.

In one embodiment, the Glu-42 or Lys-89 or both Glu-42 and Lys-89 are replaced with a non-polar amino acid, preferably alanine.

In addition, the invention provides for a DNA encoding said polypeptide, preferably encoding a polypeptide of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8.

In one embodiment, DNA encoding SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8 is fused to a signal peptide, preferably that of the hGH. Moreover, the invention also provides a vector comprising said DNA capable of expressing the polypeptide encoded by said DNA in an appropriated host cell, e.g. a prokaryotic or eukaryotic host cell.

In addition, the present invention provides for pharmaceutical compositions comprising said a polypeptide for the treatment of diseases which are prevented or alleviated by Th1 responses, preferably for treatment of viral disease or cancer.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A shows the nucleotide sequence encoding the WT IL-18 precursor and the location of primers used to construct the different mutated IL-18 proteins. The broad arrow indicates where the mature IL-18 protein coding sequence begins.

Figure 1B shows the amino acid sequence of the mature IL-18. The consensus sequence among different species of IL-18 are enclosed in the white boxes. The cystidines are underlined. The dark boxes show the residues mutated in IL-18 according to this invention.

Figure 2 shows the schematic representation of the IL-18 mutants according to the invention. The His-6 indicates the location of the six histidines fused in the N terminus of the IL-18 precursor propiece. The arrow indicates the ICE cleavage site as replaced by the factor Xa cleavage site (x). WT indicates the wild-type mature IL-18. E42A indicates Glu-42 to Ala mutation, K89A indicates Lys-89 to Ala mutation and E42A/K89A indicates double mutation. On the basis of precursor /x/ WT, three IL-18 mutants (E42A, K89A and E42A+K89A) were generated by two step PCR.

Figure 3 A shows the induction of IFN- in NKO cells by IL-18 WT and mutant protein at concentrations shown under the x-axis in Fig. 3B and in the presence of IL-12 (0.5 ng/ml)

Figure 3 B shows the induction of IFN- in PBMCs cells by IL-18 WT and mutant protein at concentrations shown under the x-axis and in the presence of IL-12 (1.0 ng/ml).

Figure 4 A shows the effect of IL-18BP in IFN- induction by human IL-18 WT and mutant protein in NKO cells. Mutants and WT IL-18 (30ng/ml) were preincubated with IL-18BP at the concentrations indicated under the x-axis (of Fig. 3B) for 1 h at room temperature and added to NKO cells stimulated with IL-12 (0.5 mg/ml).

Figure 4 B shows the effect of IL-18BP in IFN- induction by human IL-18 WT and mutant protein in PBMCs cells. Mutants and WT IL-18 (30ng/ml) were preincubated with IL-18BP at the concentrations, indicated under the x-axis for 1 h at room temperature and added to PBMCs cells stimulated with IL-12 (1.0 mg/ml).

Figure 5 shows the induction of IL-8 by IL-18 WT and mutant protein. PBMCs were incubated with IL-18 WT or mutant (30 ng/ml). Polymyxin B (1 g/ml) was mixed with IL-18 for 30 min before being added to the PBMCs. After 24 h the supernatants was removed and assayed for IL-18 concentration by ECL (example 9). One out of three experiments is shown.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to an IL-18 mutant or its active fragment, or a mutein, or any other protein or peptide derivative thereof (IL-18M), which is less susceptible to neutralization by IL18BP as compared to the wild type (IL-18 WT). More specifically, one or more amino acids of the IL-18 WT, preferably no more than 30, more preferably up to 10 amino acids, may be replaced with other amino acids, or eliminated, or one or more amino acids may be added, preferably no more than 30, more preferably up to 10 amino acid in order to generate an active IL-18 mutant which is less susceptible to neutralization by IL-18BP. Amino acids may be replaced by different amino acids the substitutions preferably being non-conservative substitutions. More specifically, said mutations could be targeted to residues predicted to be involved in binding to IL-18BP such as Glu-42, Ile-85, Met-87, Lys-

89, Met-96, Asp-130, Lys-132, Pro-143, Met-149, and Leu-189, more preferably Glu-42 and/or Lys-89 (Kim et al., 1999).

IL-18M may be produced, in eukaryotic or eukaryotic expression systems, intracellulary, periplasmic or may be secreted into the medium. The produced IL-18M may be recovered in soluble or insoluble form (inclusion bodies).

A vector comprising the precursor IL-18M cDNA may be used for expression of correct assembled precursor IL-18M in prokaryotic systems. Subsequently the mature fully active protein can be generated after cleavage by ICE *in vitro*. A sequence encoding the specific cleavage site for a protease , preferably factor Xa, can replace the ICE sequence in the precursor of IL-18M.

An expression vector encoding an effective signal peptide, preferably the human growth hormone signal peptide, fused to the cDNA of the mature IL-18M may be used for eukaryotic expression and secretion.

The parental IL-18 cDNA used for the mutant construction can be selected from mouse or human species.

IL-18M can be epitope tagged, preferably with histidine, for convenient purification. The recombinant IL-18M may be purified by conventional or affinity methods. The amount of IL-18M produced may be monitored by a specific ELISA.

IL-18M can be used in a pharmaceutical formulation for treatment of diseases which are prevented or reduced by Th1 responses, more specifically by IL-18 treatment e.g. microorganism infections and cancer. The advantage of using the mutant instead of the wild type version of the protein, resides in its resistance to IL-18BP neutralization.

More specifically, IL-18M may be administered systemically or locally as an adjuvant for tumor antigens in tumor immunoterapy.

Tumor cells derived from a patient can be isolated and genetically modified to secrete IL-18M and re-grafted to the same patient for local vaccination (Coughlin et al., 1998). Fusion of the modified tumor cells expressing IL-18M to allogeneic dendritic cells (antigen presenting cells) can be carried out to further increase the tumor antigenicity and consequently, the anti-tumor response.

IL-18M can be administered as an adjuvant in DNA vaccination (Tuting et al., 1998). In manner similar to that reported for the use of the cytokines IL-12 and IFN-. In this case, transdermal tumor antigen vaccination can be performed by using a gene gun. This may result in transfection of resident dendritic cells in the skin with DNA encoding both tumor antigen and IL-18M. Alternatively, the dendritic cells can be engineered *ex vivo* followed by an adoptive transfer.

IL-18M can be used as an adjuvant in graft versus tumor therapy. Allogeneic stem cells can be used to transplant cancer patients. To increase the graft versus tumor rejection induced by transplantation of the allogeneic cells, IL-18M may be administered systemically or locally, preferably by IL-18M expression in genetically modified syngeneic dendritic cells or in tumor cells taken from the patient.

It is to be understood that while the invention has been described in conjunction with the preferred specific embodiments thereof that the foregoing description as well as the examples that follow are intended to illustrate and not limit the scope of the invention. Other aspects, advantages and modification within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

EXAMPLES

Example 1

Construction of vectors for the expression of WT proIL-18 Histidine tag fusion protein for cleavage by Factor Xa. In order to generate the correct assembled IL-18 in *E.coli*, the ICE cleavage site in the precursor of IL-18 was replaced by a Xa cleavage site. Subsequently in vitro, cleavage of the IL-18 precursor by Xa generates therefore the active protein (WO 00/61768).

The cDNA sequence encoding the human IL-18 precursor (pro1L18, gene bank accession number D49950, Figure 1) used for generating the expression plasmid was isolated as described (Ghayur et al., 1997).

The replacement of the ICE cleavage site with a Xa cleavage site was achieved by using 2 PCR reactions (see primers used in Figure 1). PCR reaction 1: The propiece of IL-18 cDNA was generated by using the sense primer (Pr 1) containing

the EcoRI site located upstream of the ORF,
5'-ATATGAATTCATGGCTGCTGAACCAGTAG, (SEQ ID NO: 11) and a reverse primer (Pr 2) designed for the ICE site (33-LESD-36) in which 6 nucleotides have been changed to encode the factor Xa site (33-IEGR-36),
5'-AAAGTAACGTCCTTCGATGTTTTC (SEQ ID NO: 12). The amplified DNA fragment encoding the mature 1L-18 was generated by using the sense primer (Pr 3) which is complementary to Pr2,

5'-GAAAACATCGAAGGACGTTACTTT, (SEQ ID NO: 13) and the reverse primer (Pr 4) containing the BamHI site downstream of the coding sequence of IL18
5'-ATATGGATCCTAGTCTTCGTTTTGAACAGTG (SEQ ID NO: 14). The propiece 108 bp and mature 474 bp IL-18 DNAs were resolved by electrophoresis in 1% agarose, and eluted by a gel extraction system (GIBCO/ BRL).

PCR reaction 2: The two DNA fragments obtained in the PCR reaction 1 were mixed at a 1:1 ratio and used together with primers Pr 1 and Pr 4 to generate a complete human IL-18 cDNA in which the ICE site is replaced by the factor Xa site (ICE/Xa).

The pro IL-18 (ICE/Xa) cDNA was ligated into the BlueScipt plasmid (Stratagene) by EcoRI and BamHI (GIBCO/BRL) restriction sites. This plasmid served for sequence confirmation. The predicted amino acid sequence encoded is shown in SEQ ID NO:2. For *E. coli* expression, the IL-18 DNA insert was re-ligated into the pPROEX HTa vector (GIBC0/BRL) with the use of EcoRI and XbaI sites (originating in BlueScript). In this vector, the resulting protein is N-terminal fused to a histidine tag.

Example 2

Construction of the E42A, K89A and E42A/K89A mutants. Mutations in IL-18 were created in residues predicted to be important for the binding to the inhibitor IL-18BP(Kim et al. 2000). Three mutants: E42A, K89A, and F-42A/K89A, were generated. The mutations were achieved by two PCR reactions, as described in example 1, using the primers and templates described below (the primers are shown in Figure 2).

E42A mutant

PCR reaction 1- The pair of primes used for preparing the mutant E42A were: pair A- Pr 1 (example 1) and the reverse primer (Pr 5) 5'- TAA TTT AGA **TGC** AAG CTT GCC (SEQ ID NO:15) encoding alanine instead of glutamic acid (E 42), and Pair B the sense primer (Pr 6) 5'-GGC AAG CTT **GCA** TCT AAA TTA (SEQ ID NO:16) encoding Alanine in exchange for Glutamic acid (GAA to GCA) and the reverse primer Pr 4 (example 1), using pro IL-18 (ICE/Xa) as a template in the PCR reaction.

PCR reaction 2: The two DNA fragments obtained in PCR reactions 1 were used as templates for the second PCR reaction using primers Pr1 and Pr 4.

K89A mutant

PCR reaction 1: The pair of primes used for preparing the mutant K89A were pair A Pr 1(example 1) and the reverse primer (Pr 7) 5'-CTG GCT ATC **TGC** ATA CAT ACT (SEQ ID NO:17) encoding alanine instead of lysine (K 89), and pair B the sense primer (Pr 8) 5'-AGT ATG TAT **GCA** GAT AGC CAG (SEQ ID NO:18) encoding alanine instead of Lysine (AAA to GCA) with the reverse primer Pr 4 (Example 1) using pro IL-18 (ICE/Xa) as the template for the first PCR reaction.

PCR reaction 2: The two DNA fragments obtained in the PCR reaction 1 were used as templates for the second PCR reaction using primers Pr1 and Pr 4 .

E42A/K89A mutant

For the double mutation E42A/K89A, the primer used were the same as for the preparation of the F-42A mutation and mutant K89A cDNA was used as the template in the reaction.

Each of the three IL-18 mutated genes were ligated into the BlueScript vector for sequence confirmation. The predicted amino acid sequence for the precursor IL-18 E42A, K89A and E42A/K89A mutants are shown in SEQ ID NO:3, SEQ ID NO:4 SEQ ID NO:5, respectively. For ***E. coli*** expression, each of three IL-18 DNA inserts were re-ligated into the pPROEX HTa vector (GIBC0/BRL) with the use of EcoRI and XbaI sites. The resulting protein is N-terminal fused to histidine (Fig. 1).

Example 3

Protein expression and purification. The IL18 mutant precursors were expressed in E.coli, affinity purified by virtue of the histidine tag and the respective mature molecules were generated by proteolytic cleavage with factor Xa.

Each of the four pPROEX HTu/IL-18 plasmids (WT and three mutants) was introduced into competent *E. coli* cells of the DHQ strain (GIBCO/BRL) and expressed as described (11). An overnight culture of 25 ml served as the inoculum for a 450 ml of LB culture containing 100 µg/ml ampicillin and grown until it reached a cell density of 0.6-1 OD_{600}. Protein expression was induced by treatment with isopropylthiogalactoside (IPTG 0,3 mM), and incubation continued at 37°C with shaking for 3 h. The cultured bacteria cells were harvested by centrifugation (5,000 x g for 15 min at 4°C), and the pellet was suspended in 30 ml of Talon buffer (50 mM NaH_2P0_4/20 mM Tris-HCl/100 mM NaCl, pH 8). Cells were lysed by sonication (2 x 30 s bursts) on ice. The soluble protein was obtained by centrifugation (4,000xg for 30 min at 4°C) and applied to a 3 ml mini-Talon column (CLONTECH). The Talon column was then washed with 30 bed volumes of Talon buffer and eluted with 6 ml of 100 mM imidazole in Talon buffer. The eluant was dialyzed against factor Xa buffer (20 mM Tris-HCl/150 mM NaCI/2 mM CsCl2) at 4°C for 20 h. The 0.2 ml of Talon affinity-purified N-terminus His x 6 fusion proIL-18 was incubated with 4 µg of factor Xa enzyme (New England Biolabs) for 4 h at room temperature in the presence of 2 mM phenylmethylsulfonyl fluoride (GIBCO/BRL). The amount of IL-18 produced was monitored by a specific ELISA(R&D Systems). The amino acid sequence predicted for the mature IL-18 WT, E42A, K89A and E42A/K89A mutants are shown in sequences SEQ ID NO:6, SEQ ID NO:7 SEQ ID NO:8, respectively.

Example 4

Characterization of the E42A, K89A and E42A/K89A IL-18 mutant proteins by Western blot. The purified IL-18 mutants were subjected to western blot analysis with a polyclonal antibody and a monoclonal antibody specific for the mature IL-18.

Equal amounts of talon affinity purified precursor and mature protein (after cleavage by factor Xa) the WT and mutant IL-18 forms, were resolved by SDS/PAGE

(10% acrylamide) under reducing conditions. The proteins were transferred to nitrocellulose membranes and then incubated with the primary antibodies (rabbit anti-human IL-18 polyclonal antibody or monoclonal antibody clone 8-31-4 (IgG2a) which were raised against the recombinant mature form of human IL-18 (Puren et al., 1999) which also recognize precursor IL-18). After 24 h incubation, the corresponding second antibody, goat anti-mouse or donkey anti-rabbit IgG peroxidase (Jackson Immuno Research), was added and developed by ECL (New England Nuclear Life Science Products).

The staining of proIL-18 by polyclonal rabbit anti-human IL-18 was of equal intensity for the WT and each of the three mutants. Similarly the signals obtained with the mature forms of WT and IL-18 and each of the three mutants using the polyclonal antiserum were of equal intensity. The apparent molecular weigh indicated that the different IL-18 forms were of the correct size. In contrast, when the monoclonal antibody is used, the two mutants K89A and E42A/ K89A, appear to stain more intensely than the WT and the E42A mutant, suggesting that the affinity of the monoclonal antibody is greater for these mutants. These results suggest that mutants K89A and E42A/K89A may have a different conformation resulting in higher affinity.

Example 5

Characterization of the biological activity of E42A, K89A and E42A/K89A IL-18 mutant proteins. The purified mature forms of IL-18/ICE/Xa were analysed for the co-induction of IFN-γ in human natural killer cells (NKO described in example 8), in PBMCs (described in example 7) and for the induction of IL-8 in PBMCs.

IL-18 does not induce IFN-γ in these cells unless IL-12 (or IL-15) is used as a co-stimulator. Low concentrations of IL-12 (1-2 ng/ml 12 (PreproTech Rocky Hill, NJ)) induce a small amount, of IFN-γ, however, treatment with IL-12 together with IL-18 greatly augments IFN-γ production. IFN-γ produced was monitored in the cell as described in example 9. The induction of IFN-γ in NKO cells by WT IL-18/ICE/Xa and IL-12 was found to be comparable to that induced by recombinant

mature human IL-18 resulting from ICE processing of proIL-18 (Gu et al., 1997) and IL-12. These results indicate that the IL-18 was correctly assembled in *E. coli* and correctly processed by factor Xa.

To test the activity of the mutated IL-18, the induction of IFN-γ production by stimulation with the mutant or WT IL-18 together with IL-12 was assessed in NKO cells (statistical analyses are described in example 10). As shown in Fig. 3A, WT IL-18 was active as an inducer of IFN-γ , beginning at 7.5 ng/ml and increasing progressively up to 60 ng/ml (the highest concentration tested). Each of the three mutated IL-18 forms exhibited biological activity greater than that of WT in these cells. For example, the single mutation E42A was twice as active as the WT form at each of the concentrations tested. The single mutation KB9A was four times more active than the WT at a concentration of 7.5 ng/ml. The double mutation E2A/K89A resulted in the most active IL-18. As shown in Fig. 3A, the E42A mutated IL-18 induced 600 pg/ml IFNγ, the maximal activity observed by pretreatment with 60 ng/ml IL-18 WT, at a concentration of 30 ng/ml, the K89A mutant at a concentration of 15 ng/ml and the double mutant at a concentration of 7.5 ng/ml. The mutants E42A, K89A and double mutant were therefore 2, 4 and 8 folds more potent than the WT, respectively.

Similar results were observed when IFN-γ production was tested in freshly isolated human PBMCs (example 7). In these cells, the co-stimulation of IL-12 and IL-18 resulted, in IFN-γ, production, whereas neither of the two cytokines alone could induce IFNγ. The double mutant E42A/ K89A was the most active (Fig. 3B).

The results indicate that replacement of the two charged amino acids Glu 42 and/or Lys 89 by Ala residues consistently bring about an increase in the biological activity of IL-18.

IL-18 is known to induce IL-8 in CD14+ cells in PBMC preparations (described in example 7). Although IL-18 induces IL-8 production in these cells without the need of IL-12 co-stimulation, the induction of IL-8 requires higher concentrations of IL-18 than induction of IFN-γ. Induction of IL-8 by IL-18 WT and mutant stimulation of PBMCs was therefore tested. The IL-8 produced was monitored in the cell media by the specific assay described in example 9. Figure 4

shows that although the two single mutations were comparable to the WT in the induction of IL-8, the double mutated IL-18 induced significantly more IL-8 (3.5 fold) than the wild type version.

These results indicate that the double mutant, E42A/K89A exhibits the highest biological activity.

Example 6

Neutralization of Il-18 mutants by IL-18BP. The mutations were designed in residues predicted to be important for IL-18 binding by the inhibitor IL-18BP. The ability of IL-18BP to neutralize the biological activity of IL-18, e.g IFN-γ production (example 8), was therefore specifically assessed.

Different concentrations of IL-18BP ("a" isoform of CHO cell produced recombinant his-6-tagged human IL-18BP (supplied by Interpharm Laboratories, Ness Ziona, Israel Kim et al., 2000)) were pre-incubated with WT IL-18 or its mutated forms (30 ng/ml final concentration) and then added to cell cultures.

As shown in Fig. 4A, the 50% inhibitory concentration of IL-18BP for co-induction of IFN-γ by WT IL-18 from NKO cells was approximately 15 ng/ml (assuming that no inhibition occurs at 3.7 ng/ml IL-18BP and this value represent 100% activity). The single mutation of E42A resulted in a similar dose-inhibitory concentration by IL-18BP.

However, when the mutant K89A was incubated with IL-18BP, its ability to act as a co-inducer of IFN-γ in NKO cells was neutralized at a lesser extent (Fig. 5A). Only at a concentration of 120 ng/ml a statistically significant reduction in activity could be observed. In contrast, IL-18BP failed to neutralize the double IL-18 mutant E42A/K89A.

As shown in Fig. 4B IL-18 is more sensitive to neutralization by IL-18BP when tested in PBMCs rather than NKO cells. The amount of IL-18BP needed to neutralize WT IL-18 was 3.7 ng/ml, the lowest concentration tested. The single mutation E42A behaved similarly as WT IL-18, as established by the observation that low concentrations of IL18BP neutralized its biological activity in PBMCs. In contrast, the single mutation K89A was neutralized at 120 ng/ml. Similar to the

results concerning neutralization of IL-18 mutants by IL-18BP in NKO cell, the double mutant E42A/ K89A was only slightly affected by IL18-BP in PBMCs.

These results show that the mutant E89A and the double mutant E42A/K89A are less affected by the natural inhibitor IL-18BP.

Example 7

Isolation and culture of peripheral blood mononuclear cells (PBMCs) and induction of IFN-γ. Residual leukocytes from platelet plateletpheresis of healthy human donors were rinsed from blood tubing and subjected to centrifugation over Histopaque. PBMCs were aspirated from the interface, washed three times in pyrogen-free saline (Baxter Health Care, Mundelein, IL), and resuspended at 5 x 10^6 cells per ml in RPMI 1640 medium supplemented with 10% FBS (GIBCO/BRL Grand Island, NY). The cells were cultured in flat-bottomed 96-well plates (Becton Dickinson) with RPMI 1640 medium only (control), varying concentrations of recombinant human IL-18, and WT IL-18 (ICF/Xa) or the three mutants, in the presence of 1 ng/ml IL-12. In some experiments, IL18 preparations were first mixed with polymyxin B (1 μg/ml purchased from Sigma) before being added to the cells. Cells were incubated for 16-20 h at 37°C in humidified air with 5% CO_2, and the culture supernatant was then collected for IFN-γ measurement.

Example 8

Induction of IFN-γ in NKO Cell Line. The original parental NK92 cell line was obtained from Hans Klingerman (Gong et al., 1994). The human NKO cell line used in the present studies was a subclone of this cell line. NKO cells were maintained in supplemented RPMI 1640 medium containing 10% FBS and 50 pg/ml of IL-2 (R&D Systems) and 200 pg/ml of IL-15 (PeproTech). For assays, NKO sells were suspended at 0.5 x 10^6 cells per ml in RPMI 1640 medium and stimulated in 0.2 ml volumes in 96-well plates with 0.5 ng/ml of IL-12 (PreproTech Rocky Hill, NJ) and different concentrations of recombinant human IL-18 WT, IL-18 (ICE/Xa), or E42A, K89A and E42A/K89A IL-18 mutants. After 16-20 h at 37°C in humidified air with 5% CO_2, the culture supernatant was collected for IFN-γ measurement.

Example 9

Analysis of Cytokines. The liquid-phase electrochemiluminescence (ECL) method was used to measure IFN-γ (13) and IL-8 (12) in cell culture media. The amount of ECL was determined with the use of an Origen Analyzer (Igen, Gaithersburg, MD). The limit of detection of IFN-γ and IL-8 was 62 pg/ml and 40 pg/ml, respectively.

Example 10

Statistical analysis. Data are expressed as the mean ± SEM. Group means were compared by ANOVA, with the use of Fisher's least significant difference. Statistical significance was accepted within 95% confidence limits. ANOVA and correlation analyses were performed with the statistical packages STATVIEW 512 +(Brain Power, Calabasas, CA).

Example 11

Production of the mature IL-18 mutants in CHO cells. For expression and secretion of mature IL-18 mutants in CHO cells, the DNA sequence encoding the mature protein of wild type and mutant IL-18BP is ligated to the sequence of the DNA sequence of the signal peptide of human growth hormone (hGH) by two PCR reactions similarly to the reactions described in example 1. The template for the first PCR reaction for the amplification of each IL-18 mutant is the corresponding construct from example 2 with sense primer (Pr 9) containing overlapping sequences of IL-18 and hGH signal peptide and reverse primers (Pr 10) encoding the last 12 nucleotides of the IL-18, a stop codon and a site for a restriction enzyme. For the amplification of the growth hormone signal peptide the plasmid pXGH is used as the template with a sense primer (Pr 11) containing a site for a restriction enzyme, the first 12 nucleotides of the hGH signal peptide and the reverse primer (Pr 12), containing overlapping sequences with the hGH signal peptide and IL-18 mature protein. The templates for the second PCR performed for the amplification of the fragment encoding the signal peptide of the hGH fused to the mature sequence of the

IL-18, are the purified amplified fragments from the first PCR reaction and the primers Pr 10 and Pr 11 containing the restriction sites. The fusion fragment is purified, digested with the appropriate restriction enzymes and cloned into a mammalian expression vector.

The plasmids are used for transfecting CHO (DHFR-) cells together with a plasmid containing the mouse DHFR gene as a genetic marker. Resistant cells are isolated in a selective medium and assayed for IL-18 production by an ELISA assay.

The stably transfected cells are subjected to several cycles of gene amplification with increasing concentrations of MTX. At the end of the gene amplification process, clones are isolated by limiting dilution. After subcloning the clone that show high specific productivity and grater stability of production is selected for production.

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The references cited above and throughout the specification are herein incorporated by reference in their entirety.

CLAIMS

We claim:

1. An IL-18 mutant polypeptide, comprising mutations in one or more amino acid residues which are involved in its interaction with IL-18 binding protein.

2. The polypeptide according to claim 1, wherein the mutations are substitutions, additions or deletions.

3. The polypeptide according to claim 2, wherein the substitutions are non-conservative.

4. The polypeptide according to anyone of claims 1, 2 or 3, wherein the mutation is in a residue selected from a group consisting of Glu-42, Ile-85, Met-87, Lys-89, Met-96, Asp-130, Lys-132, Pro-143, Met-149, and Leu-189.

5. The polypeptide according to claim 4, wherein the mutation is in a residue selected from the group consisting of Glu-42 and Lys-89.

6. The polypeptide according to claim 5, wherein the mutation is in a Glu-42 residue.

7. The polypeptide according to claim 5, wherein the mutation is in a Lys-89 residue.

8. The polypeptide according to claim 5, wherein the mutation is in a Glu-42 residue and a Lys-89 residue.

9. The polypeptide according to claim 6, wherein the Glu-42 residue is replaced with a non-polar amino acid.

10. The polypeptide according to claim 9, wherein the Glu-42 is replaced with an Ala residue.

11. The polypeptide according to claim 7, wherein the Lys-89 residue is replaced with a non-polar amino acid.

12. The polypeptide according to claim 11, wherein the Lys-89 is replaced with an Ala residue.

13. The polypeptide according to claim 8, wherein both the Glu-42 residue and the Lys-89 residues are replaced with a non-polar amino acid.

14. The polypeptide according to claim 13, where both the Glu-42 and the Lys-89 residues are replaced with an Ala residue.

15. An isolated DNA encoding a polypeptide according to any one of claims 1-14.

16. The DNA according to claim 15, wherein the polypeptide has amino acid sequence of SEQ ID NO:3.

17. The DNA according to claim 15, wherein the polypeptide has amino acid sequence of encoding the polypeptide of SEQ ID NO:4.

18. The DNA according to claim 15, wherein the polypeptide has amino acid sequence of encoding the polypeptide of SEQ ID NO:5.

19. The DNA according to claim 15, wherein the polypeptide has amino acid sequence of encoding the polypeptide of SEQ ID NO:6.

20. The DNA according to claim 15, wherein the polypeptide has amino acid sequence of encoding the polypeptide of SEQ ID NO:7.

21. The DNA according to claim 15, wherein the polypeptide has amino acid sequence of encoding the polypeptide of SEQ ID NO:8.

22. A DNA according to any one of claims 15, 19, 20 and 21 further comprising a nucleic acid sequence encoding a signal peptide.

23. The DNA according to claim 22, wherein the signal peptide is that of a growth hormone.

24. A vector comprising a DNA according to any one of claims 15 to 23, wherein said vector is capable of expressing the polypeptide encoded by said DNA in an appropriate host cell.

25. The vector according to claim 24, wherein the host cell is prokaryotic.

26. The vector according to claim 25, wherein the DNA encodes a polypeptide selected from a group consisting of SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5.

27. The vector according to claim 24, wherein the host cell is an eukaryotic cell.

28. The vector according to claim 27, wherein the DNA encodes a polypeptide selected from a group consisting of SEQ ID NO:6, SEQ ID NO:7 and SEQ ID NO:8.

29. The vector according to claim 27, comprising DNA according to claim 22 or 23.

30. The vector according to claim 28, wherein said DNA is ligated to the sequence encoding the human growth hormone signal peptide.

31. A pharmaceutical composition comprising a polypeptide according to any one of claims 1 to 14 and a pharmaceutically acceptable carrier for the treatment of diseases which are prevented or alleviated by Th1 responses.

32. The pharmaceutical composition according to claim 31, for the treatment of cancer.

33. The pharmaceutical composition according to claim 31, for the treatment of viral diseases.

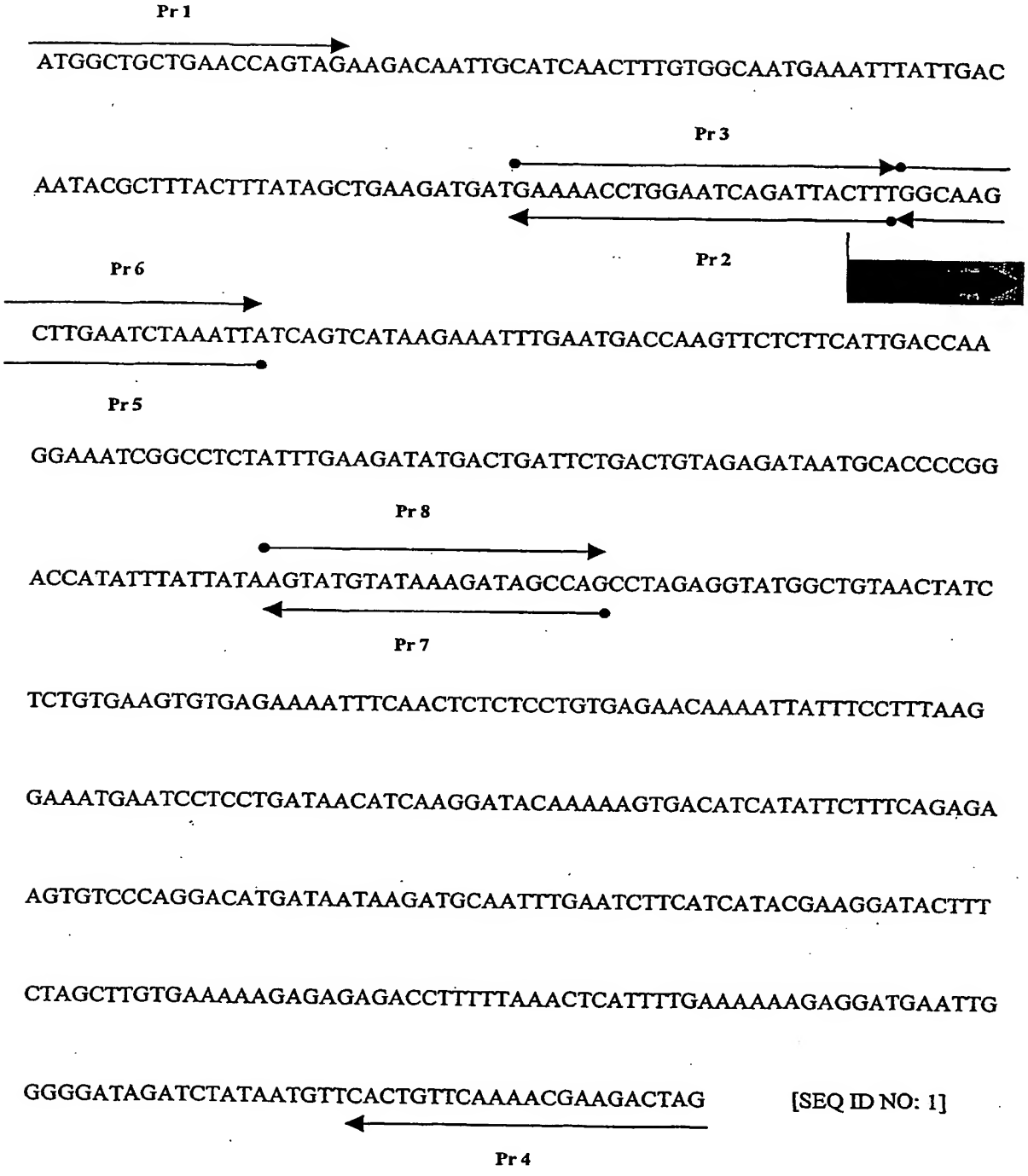
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Figure 1 A

Tyr	Phe	Gly	Lys	Leu	Glu	Ser	Lys	Leu	Ser	Val	Ile	Arg	Asn	Leu	Asn
1				5					10					15	
Asp	Gln	Val	Leu	Phe	Ile	Asp	Gln	Gly	Asn	Arg	Pro	Leu	Phe	Glu	Asp
			20					25					30		
Met	Thr	Asp	Ser	Asp	Cys	Arg	Asp	Asn	Ala	Pro	Arg	Thr	Ile	Phe	Ile
		35					40					45			
Ile	Ser	Met	Tyr	Lys	Asp	Ser	Gln	Pro	Arg	Gly	Met	Ala	Val	Thr	Ile
	50					55					60				
Ser	Val	Lys	Cys	Glu	Lys	Ile	Ser	Thr	Leu	Ser	Cys	Glu	Asn	Lys	Ile
65					70					75					80
Ile	Ser	Phe	Lys	Glu	Met	Asn	Pro	Pro	Asp	Asn	Ile	Lys	Asp	Thr	Lys
				85					90					95	
Ser	Asp	Ile	Ile	Phe	Phe	Gln	Arg	Ser	Val	Pro	Gly	His	Asp	Asn	Lys
			100					105					110		
Met	Gln	Phe	Glu	Ser	Ser	Ser	Tyr	Glu	Gly	Tyr	Phe	Leu	Ala	Cys	Glu
		115					120					125			
Lys	Glu	Arg	Asp	Leu	Phe	Lys	Leu	Ile	Leu	Lys	Lys	Glu	Asp	Glu	Leu
	130					135					140				
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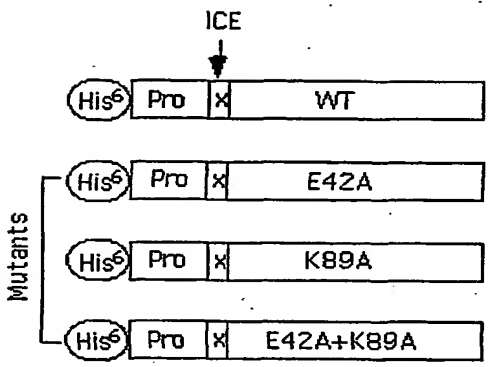
Figure 1 B



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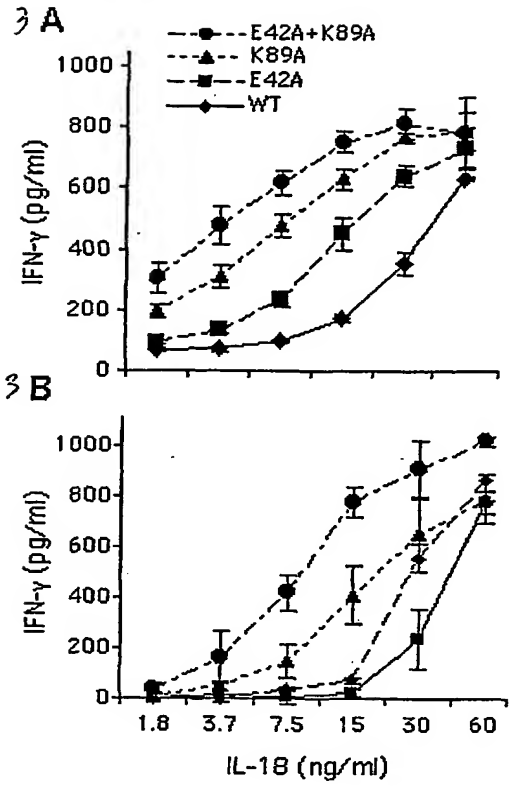
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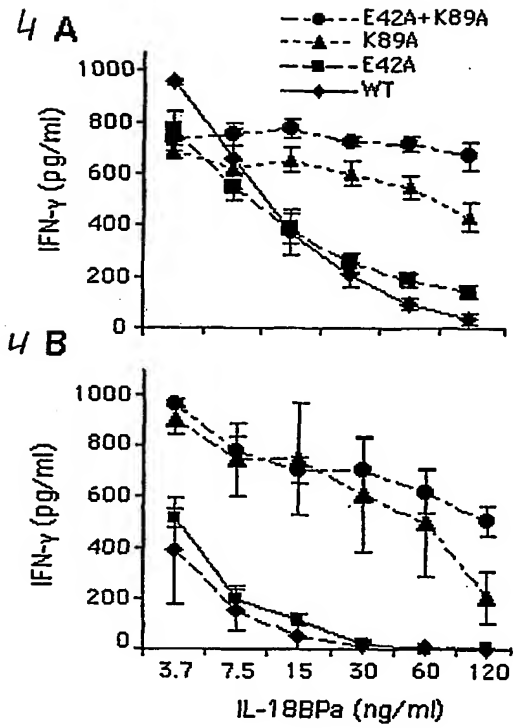
E42A+K89A
K89A
E42A
WT
IFN-γ (pg/ml)
1000
800
600
400
200
0
3 B
IFN-γ (pg/ml)
1000
800
600
400
200
0
1.8
3.7
7.5
15
30
60
IL-18 (ng/ml)

Figure 3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30
60
120
IL-18BPa (ng/ml)

Figure 4

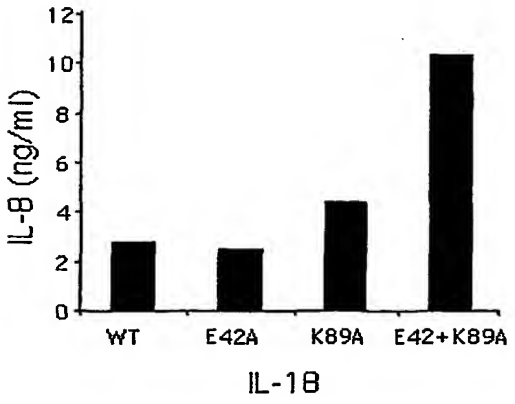

Figure 5

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Kim, Soo Hyun

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Met Thr Asp Ser Asp Cys Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
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Ile Ser Met Tyr Lys Asp Ser Gln Pro Arg Gly Met Ala Val Thr Ile
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Ser Val Lys Cys Glu Lys Ile Ser Thr Leu Ser Cys Glu Asn Lys Ile
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Leu Phe Glu Asp Met Thr Asp Ser Asp Cys Arg Asp Asn Ala Pro Arg
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100 105 110

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Lys Asp Thr Lys Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly
130 135 140

His Asp Asn Lys Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe
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Arg Asn Leu Asn Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro
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Lys Asp Thr Lys Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly
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Arg Asn Leu Asn Asp Gln Val Leu Phe Ile Asp Gln Gly Asn Arg Pro
50 55 60

Leu Phe Glu Asp Met Thr Asp Ser Asp Cys Arg Asp Asn Ala Pro Arg
65 70 75 80

Thr Ile Phe Ile Ile Ser Met Tyr Ala Asp Ser Gln Pro Arg Gly Met
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Ala Val Thr Ile Ser Val Lys Cys Glu Lys Ile Ser Thr Leu Ser Cys
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Glu Asn Lys Ile Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile
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Lys Asp Thr Lys Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly
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His Asp Asn Lys Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe
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Ser Val Lys Cys Glu Lys Ile Ser Thr Leu Ser Cys Glu Asn Lys Ile
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Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
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Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Cys Glu
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Ser Val Lys Cys Glu Lys Ile Ser Thr Leu Ser Cys Glu Asn Lys Ile
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Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
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Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
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Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Cys Glu
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Lys Asp Thr Lys Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly
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His Asp Asn Lys Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe
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Met Thr Asp Ser Asp Cys Arg Asp Asn Ala Pro Arg Thr Ile Phe Ile
35 40 45

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Ser Val Lys Cys Glu Lys Ile Ser Thr Leu Ser Cys Glu Asn Lys Ile
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Ile Ser Phe Lys Glu Met Asn Pro Pro Asp Asn Ile Lys Asp Thr Lys
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Ser Asp Ile Ile Phe Phe Gln Arg Ser Val Pro Gly His Asp Asn Lys
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Met Gln Phe Glu Ser Ser Ser Tyr Glu Gly Tyr Phe Leu Ala Cys Glu
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(74) Agent: SERONO INTERNATIONAL S.A.; Intellectual Property, 12, chemin des Aulx, CH-1228 Plan-les-Ouates (CH).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
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18 December 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/101049 A3

(54) Title: INTERLEUKIN-18 MUTANTS, THEIR PRODUCTION AND USE

(57) Abstract: The invention provides mutants of IL-18 with lower affinity to IL-18BP than the wild type IL-18 molecule.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 02/02344

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/24 C07K14/54 A61K38/20 A61P31/12 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, WPI Data, PAJ, MEDLINE, EMBL, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	KIM SOO-HYUN M ET AL: "Site-specific mutations in the mature form of human IL-18 with enhanced biological activity and decreased neutralization by IL-18 binding protein." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 98, no. 6, 13 March 2001 (2001-03-13), pages 3304-3309, XP002226879 March 13, 2001 ISSN: 0027-8424 the whole document ---	1-33
Y	EP 0 845 530 A (HAYASHIBARA BIOCHEM LAB) 3 June 1998 (1998-06-03) cited in the application the whole document ---	1-33
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
13 January 2003	31/01/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Bucka, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	KIM S-H ET AL: "STRUCTURAL REQUIREMENTS OF SIX NATURALLY OCCURRING ISOFORMS OF THE IL-18 BINDING PROTEIN TO INHIBIT IL-18" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 97, no. 3, 1 February 2000 (2000-02-01), pages 1190-1195, XP000941910 ISSN: 0027-8424 cited in the application page 1193, right-hand column; figure 6 ---	1-33
Y	RUBINSTEIN M ET AL.: "Cytokines, cytokine receptors and cytokine inhibitors" LIFE SCIENCE BOOK 2000, 'Online! 2000, XP002226976 WEIZMANN INSTITUTE OF SCIENCE, REHOVOT, ISRAEL Retrieved from the Internet: <URL:http://www.weizmann.ac.il/Biology/open-day/images/rubinm.pdf> 'retrieved on 2003-01-13! page 98 -page 99; figure 2 & "Life Science Book 2000" 2000 , WEIZMANN INSTITUTE OF SCIENCE , REHOVOT, ISRAEL ---	1-33
A	VIGERS GUY P A ET AL: "Crystal structure of the type-I interleukin-1 receptor complexed with interleukin-1-beta." NATURE (LONDON), vol. 386, no. 6621, 1997, pages 190-194, XP001109468 ISSN: 0028-0836 cited in the application the whole document ---	1-33
A	DINARELLO C A ET AL: "OVERVIEW OF INTERLEUKIN-18: MORE THEN AN INTERFERON-GAMMA INDUCING FACTOR" JOURNAL OF LEUKOCYTE BIOLOGY, FEDERATION OF AMERICAN SOCIETIES FOR EXPERIMENTAL, US, vol. 63, no. 6, June 1998 (1998-06), pages 658-664, XP000973451 ISSN: 0741-5400 the whole document -----	1-33

SDOCID: <WO_____02101049A3_I_>

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		EP 0845530 A2	03-06-1998
		US 6476197 B1	05-11-2002